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Microbial Synthesis and Characterization of Poly (3-Hydroxybutyrate-*co*-4-Hydroxybutyrate) Copolymers

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The research is focused on the prospective representative of the family of biodegradable polymers – polyhydroxyalkanoates (PHAs), the copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB). Difficulties with producing this type of PHA are associated with the fact that the precursor for the 4-hydroxybutyrate (4HB) monomers synthesis, the γ-butyrolactone, is toxic for the bacteria producers and reduces the total biomass production and the polymer yield when it is added to the medium. Having used the natural strains of hydrogen-oxidizing bacteria Ralstonia eutropha B5786 and Cupriavidus eutrophus B10646, the latter possessing the increased sustainability to the γ-butyrolactone influence, the conditions for efficient synthesis of P(3HB-co-4HB) have been determined. A set of highly purified samples of P(3HB-co-4HB) with different content of 4HB (from 8.7 to 24.3 mol %) was produced. It has been determined that the incorporation of 4HB in the copolymer of a higher degree than 3-hydroxyvalerate and 3-hydroxyhexanoate leads to the decrease of the copolymer's crystallinity; samples with the crystallinity of 12 % and 25 % have been obtained. It has been demonstrated that the average molecular weight of the P(3HB-co-4HB) samples and the polydispersity do not depend on the ratio of monomers and vary within the wide range from 540 to 1100 kDa and from 1.91 to 2.76 correspondingly. It has been determined that the 4HB content in the studied range (8.7-24.3 mol %) does not effect the melting temperature of the copolymers (168.9-172.5 ºC).

Keywords: hydrogen-oxidizing bacteria; poly(3-hydroxybutyrate-co-4-hydroxybutyrate); γ-butyrolactone; physicochemical properties.

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Introduction

Synthesis of the storage polymers – hydroxy derivatives of alkanoic acids polyhydroxyalkanoates (PHAs) – by microorganisms has become an extensively studied subject. PHAs are represented by various polyesters, consisting of homogenous monomers with different length of the carbon chain and by copolymers; there are high-crystallinity thermoplastic PHAs and thermolabile rubber-like elastomers (Philip et al., 2007).

Synthesis of PHAs with tailored properties is a difficult technological task, and production of a PHA of definite composition should be based on fundamental knowledge of how a certain PHA is synthesized and how the chemical structure of the polymer influences its physicochemical properties. There are several ways to produce PHAs consisting of monomers with different length of the carbon chain: using genetically modified PHA producing strains, with genes controlling the key enzymes of PHA synthesis (β-ketothiolase, acetoacetyl-CoA reductase, PHA-synthase) taken from microorganisms of different taxa and introduced into them, and based on the knowledge of the mechanisms of PHA synthesis by natural producers, which are influenced by culture conditions.

A very promising but insufficiently studied PHA is the poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)) copolymer. PHAs of this type are biodegraded *in vivo* and in the environment at high rates and exhibit better elongation at break and higher tensile strength than most of the well-known polymers of this class (Martin, Williams, 2003; Freier, 2006; Chanprateep, 2010). The ability of such microorganisms as *Cupriavidus necator* (previously known as *Wautersia eutropha, Ralstonia eutropha*, *Alcaligenes eutrophus*) (Nakamura et al., 1992), *Alcaligenes latus* (Hiramitsu et al., 1993), *Comamonas testosteronii* (Renner et al., 1996), *Comamonas acidovorans* (Saito, Doi, 1994), *Hydrogenophaga pseudoflava* (Choi et al., 1999), *Chromobacterim sp* (Kimura et al., 1999), *Rhodococcus rubber* (Haywood et al., 1991), *Delftia acidovorans* (Hsieh et al., 2009) to synthesize PHAs of this type when grown on the media containing 4-hydroxybutyric acid, γ-butyrolactone or 1,4-butanediol as carbon substrate was reported in a number of studies in the 1990s. The inhibiting effect of these substrates, however, reduces both total biomass production and copolymer yields.

In recent years, this representative of PHAs has received much attention. Recombinant *Escherichia coli*, *Aeromonas hydrophila*, *Pseudomonas putida*, *Ralstonia eutropha* H16 (Zhang et al., 2009; Li et al., 2010) and wild-type strains *Cupriavidus* sp. USMAA1020 (Amirul et al., 2008), *R. eutropha* A-04 (Chanprateep et al., 2008), *Cupriavidus* sp. USMAA2-4 (Ramachandran et al., 2011), *R.eutropha* KCTC 2662 (Park et al., 2011) have been described as novel producers of P(3HB-*co*-4HB).

The objective of this study was to research the conditions of the P(3HB-co-4HB) synthesis by natural strains of hydrogen-oxidizing bacteria and determined the effect of the composition on the properties of the copolymer and the resulted products.

Materials and Methods

Two strains of hydrogen-oxidizing bacteria – *Ralstonia eutropha* B5786 (Stasishina, Volova, 1992) and *Cupriavidus eutrophus* B10646 (registered in the Russian Collection of Industrial Microorganisms (VKPM)) were batch cultured in 1.0-L glass flasks, which were 50 % filled with the culture, under strict aseptic conditions, on a "New Brunswick" temperature-controlled shaker (USA); the cultures were grown on Schlegel mineral salt medium. Autotrophic cultures were grown on the (CO_2, H_2, O_2) gas

mixture as carbon and energy source. Air was evacuated from the flasks with a compressor, and the gas mixture was supplied from a gasholder, via airtight hoses. The proportions of CO_2, O_2 and H_2 in the gas mixture were 1:2:7 (v/v). Heterotrophic cultures were grown on fructose or butyric acid. Cells were cultured in two phases – in nitrogen-limited medium in the first and nitrogen-free one in the second, at pH 7.0 and temperature 30 °C, in accordance with our previously developed procedure (Volova et al., 1992). Synthesis of monomers 4-hydroxybutyrate (4HB) was induced by the addition of γ-butyrolactone (Fluka). The culture was added with γ-butyrolactone with varied frequency and in varied amounts. The duration of the cultivation depended on carbon nutrition conditions and varied from 72 h to 96 h.

Biomass yield was determined from dry mass and optical parameters of the culture. Polymer content and PHA composition was determined with a gas chromatograph-mass spectrometer (GC/MS, model GCD Plus, Hewlett Packard, USA) Percentages of the monomers in (P(3HB-*co*-4HB) were quantified based on mass spectra, and more accurate quantification was attained by taking ^{1}H-NMR spectra of copolymer solutions in $CDCl_3$ using an Avance III 600 NMR spectrometer (Bruker, Germany). Current concentrations of salts of alkanoic acids and γ-butyrolactone in the culture were analyzed using gas chromatography. Fructose concentration in the medium was measured using the resorcinol method (Ermakov et al., 1972).

X-ray structure analysis and determination of crystallinity of PHA samples were performed using a D8 Advance X-ray spectrometer (Bruker, Germany) (graphite monochromator on a reflected beam) in a scan-step mode, with a 0.04° step and exposure time 2 sec, to measure intensity at point. The instrument was operating at 40 kV × 40 μA. Thermal properties of biopolymers were examined using differential scanning calorimetry with a NETZSCH analyzer (Germany). Each sample of mass ~2 mg was shaped as a disc of diameter 5 mm, inserted into an aluminum capsule with a small hole, and heated at 5 K/min. Melting point, devitrification temperature, and thermal degradation temperature were determined from DSC curves at the given heating rate.

Molecular weight was determined by gel permeation chromatography at a temperature of 40 °C, using a Waters chromatographic system (a Waters 151 isocratic pump, a Reodyne 7725i injector, and a Waters 2414 refractometric detector) and columns Styragel HR4E and HR5. Chloroform was used as eluent, with the flow rate 0.8 ml/min. The system was calibrated using low polydispersity polysterene standards supplied by Sigma (USA).

The number-average molecular weight of the PHA was determined as

$$M_n=\Sigma\ (N_i \cdot M_i / N),$$

where N_i is the number of molecules of mass I; N is the total number of molecules; M_i is the mass of molecules of length I.

The weight average molecular weight of the PHA was determined as

$$M_w = \Sigma\ (w_i \cdot M_i),$$

where w_i is the portion of the mass ($w_i = N_i M_i / \Sigma\ (N_i \cdot M_i)$).

Polydispersity, which provides an estimate of the proportions of fragments with different polymerization abilities in the polymer, was calculated from the formula

$$PD = M_w / M_n.$$

Experiments were done in three replicates. The results were analyzed statistically by

conventional methods, using the standard software package of Microsoft Excel.

Results

In the autotrophic culture of *R. eutropha* B5786 grown in flasks on a shaker under nitrogen deficiency, using single-carbon substrate (CO_2), the biomass yield and the polymer content after 72-h fermentation amounted to 6.1 g/L and 65.4 % of dry cell weight (DCW), respectively (Table 1). The polymer synthesized by *R .eutropha* B5786 on single-carbon substrate contained 99.2 mol % of 3HB and 0.8 mol % of 3-hydroxyvalerate (3HV). Previously we reported that cultivation of bacteria in flasks for longer periods and the use of advanced fermentation equipment can increase polymer yield to 85-90 % of DCW (Volova, Kalacheva, 2005). The main purpose of this study, however, was to find out whether this strain is able to synthesize a PHA containing 4-hydroxybutyrate (4HB) and what growth conditions are necessary for this. Biomass yield was considered as a secondary issue, so the duration of experiments with autotrophic culture in flasks did not exceed 70 h.

Taking into account the data on the toxicity of γ-butyrolactone (Nakamura et al., 1992), we studied the effect of this supplementary substrate on *R. eutropha* B5786 growth and PHA synthesis using γ-butyrolactone concentration from 0.5 g/L to 4.0 g/L. Results of PHA biosynthesis and incorporation of 4HB into the copolymer, using mixed carbon source (CO_2 + γ-butyrolactone) are shown in Table 1: γ-butyrolactone inhibited polymer synthesis and accumulation. Higher concentrations of γ-butyrolactone in the medium did not increase 4HB proportion in the copolymer; moreover, they inhibited transformation of γ-butyrolactone to 4HB. While with 0.5 g/L of γ-butyrolactone in the medium the degree of its transformation into 4HB reached 0.21, at concentration of 4 g/L it dropped below 0.02.

The amount of γ-butyrolactone added to the culture did not affect biomass yield, which was not higher than 5.0 g/L, or 25 %-30 % lower than in the control culture (with CO_2 as sole substrate). Investigation of the influence of γ-butyrolactone on polymer synthesis showed a dose-dependent reduction in the intracellular concentration of the polymer; the highest polymer yield (39.9 % of DCW) was attained in the experiment with a single addition of 0.5 g/L of γ-butyrolactone. Polymer synthesized under these conditions contained three monomers: 3HB as a major component, 4HB, and minor amounts of 3HV. Monomer 4HB was detected in all samples, with the highest percentage amounting to 6.3 mol % (Table 1).

To reduce the toxic effect of γ-butyrolactone on the culture, it was added in several portions. By varying the size of the dose of γ-butyrolactone added to the culture and the duration of the subsequent cultivation, we managed to increase the total yield of bacterial cells and the polymer yield. When the γ-butyrolactone dose (6 g/L) was divided into two portions, the polymer yield reached 55.7 % of DCW and the total culture productivity was not decreased, but the 4HB fraction did not grow, amounting to not more than 6 mol %. When the dose of γ-butyrolactone of 9.0 g/L was divided into three portions (3 g/L each) and added every 8 h, the polymer yield was 50.3 % of DCW, the total biomass yield 4.0 g/L, and the 4HB fraction did not rise above 6 mol % (Table 1).

In the culture of *R. eutropha* B5786 grown heterotrophically, with fructose as the main substrate, the inhibitory effect of γ-butyrolactone was less pronounced (Table 1). Doses of γ-butyrolactone added in one portion were 2.0, 6.0 and 10.0 g/L. Inhibition of cell growth was only recorded with the highest concentration of γ-butyrolactone: biomass yield was as low as 4.5 g/L. At the two other concentrations,

Table 1. Content and composition of polyhydroxyalkanoates synthesized by bacteria *Ralstonia eutropha* B5786 in different conditions (experiments were done in three replicates)

Concentration of γ-butyrolactone, g/l	Hours	Biomass yield, g/l	Polymer content, % of DCW	Polymer composition, mol %		
				3HB	3HV	4HB
Autotrophic conditions (CO_2)						
0	72	6.1±0.2	65.4±4.2	99.2	0.8	-
0.5	72	4.2±0.3	39.9±1.7	93.3	0.4	6.3
1.5	72	4.3±0.2	24.9±1.9	93. 6	0.4	6.0
2.0	72	4.6±0.2	13.9±0.7	95.7	0.5	3.8
4.0	72	4.4±0.4	12.0±0.9	98.7	0.7	0.6
6.0 (3+3)*	72	5.9	55.7	93.4	0.7	5.9
9.0 (3+3+3)**	72	6.0	50.3	93.5	0.6	5.9
Heterotrophic conditions (fructose)						
2.0	96	7.5±0.4	57.3±3.4	98.6	0.5	0.9
6.0	96	7.0±0.3	50.6±4.1	97.9	0.7	1.4
10.0	96	4.5±0.3	56.7±2.9	98.0	0.4	1.6
6.0 (3.0+3.0)*	96	7.4	78.1	95.6	0.6	3.8
9.0 (3.0+3.0+3.0)**	96	7.2	65.9	95.3	0.6	4.1
Heterotrophic conditions (butyric acid)						
6.0 (3.0+3.0)**	96	7.1	81.7	95.1	0.5	4.4

* – γ-butyrolactone adding at 24 and 32 hours of cultivation
** – γ-butyrolactone adding at 24, 32 and 40 hours of cultivation

biomass yield was similar to that in the control, reaching 7.0-7.5 g/L, after cultivation for 96 h. Assimilation of γ-butyrolactone occurred at a higher rate than in the autotrophic culture, and in 34 h after γ-butyrolactone was added, its concentration in the culture did not exceed 0.1-0.2 g/L. In the experiment with the inhibitory concentration of γ-butyrolactone, polymer yield amounted to 56.7 % of DCW, but the 4HB fraction of the polymer was small – 1.6 mol %. With 6.0 g/L and 2.0 g/L of γ-butyrolactone added to the culture, polymer yield amounted to 50.6 % and 57.3 % of DCW, but the polymer contained less than 2 mol % of 4HB. In experiments with γ-butyrolactone added in several portions, polymer yield was increased to 65.9-70.1 % of DCW, but the 4HB fraction grew insignificantly – to 3.8-4.1 mol % (Table 1).

The use of butyric acid (butyric acid sodium salt) instead of fructose increased copolymer yield and improved its composition. As butyric acid concentrations above 4.0 g/L inhibited cell growth, the concentration of butyric acid was 1-2 g/l. Cultivation of *R. eutropha* B5786 on the medium containing butyric acid and 6 g/L of γ-butyrolactone (added in two portions) resulted in polymer yield of 81.7 % of DCW. The fraction of 4HB, however, remained low – not higher than 4.4 mol % (Table 1).

Thus, by varying the conditions of carbon nutrition, including the main carbon substrate and its concentration, and the mode of feeding the supplementary substrate (γ-butyrolactone) into the culture, we managed to maximize the yields of the polymer and total biomass of *R. eutropha* B5786 however the content of 4HB in copolymer was low.

Further experiments were done with the strain *C. eutrophus* B10646 which has the increased sustainability to γ-butyrolactone is compared to *R. eutropha* B5786. The highest concentration of γ-butyrolactone for this strain is 15-20 g/L. In the culture of *C. eutrophus* B10646 grown autotrophically and heterotrophically, a higher content of 4HB in the copolymer was obtained (up to 15 mol %), which on the background of the higher indexes of biomass yield and total yield of the copolymer made 6-7 g/L and 81-85 %, correspondingly.

The mode of growing *C. eutrophus* B10646 in the medium containing the propionic acid as the main substrate in the concentration 2 g/L and γ-butyrolactone provided synthesis of three component polymer containing 8.1 mol % of 4HB and 35.2 mol % of 3HV besides 3HB. Using butyric acid with γ-butyrolactone as the carbon source resulted in higher production of polymer in the culture of *C. eutrophus* B10646 (Fig. 1). Biomass yield and polymer content varied depending on the γ-butyrolactone concentration. The yields were 6.2-8.2 g/L and 72-89.7 % of DCW, correspondingly; whereas the content of 4HB varied in the range of 8.7 – 24.3 mol %. Using the strain of *C. eutrophus* B10646 with the higher tolerance to the γ-butyrolactone, effect of both the copolymer P(3HB-*co*-4HB) strain and the single-carbon substrate allowed not only to increase the content of 4HB in the copolymer, but also to reduce the time of bacteria cultivation.

Growing of *C. eutrophus* B10646 bacteria in the medium contained the butyric acid (or fructose) as the main substrate and addition of γ-butyrolactone in various concentration (from 2.0 to 10.0 g/L) allowed to fulfil the process with the total yields of biomass and copolymer within 29-32 hours of cultivation, i.e., up to 25-32 g/L and 88-95 % of DCW, with 15-20 mol % of 4HB in the copolymer. Composition of the produced and purified samples of P(3HB-*co*-4HB) were analyzed by the ^{1}H-NMR method which proved the presence of 4HB in the copolymer. The ^{1}H-NMR spectra of the copolymer samples with 4HB incorporation at 8.7 mol % and 16 mol % are presented in Fig. 2.

Thus, the substitution of the strain *R. eutropha* B5786 by the strain *C. eutrophus* B10646 and the developed mode of proportions

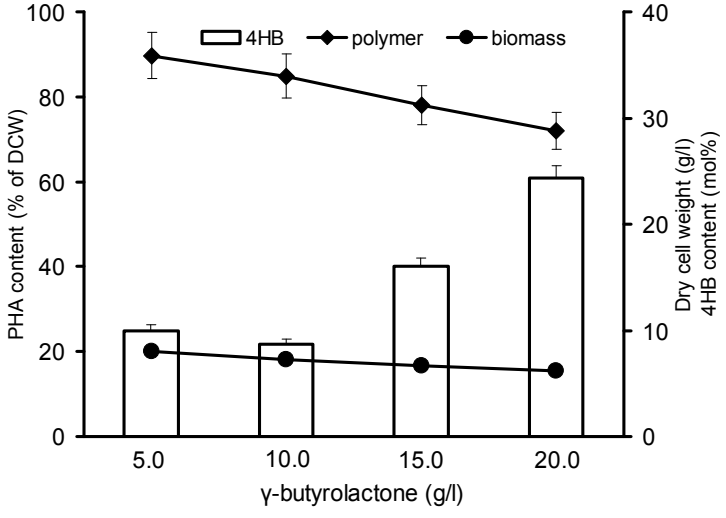

Fig. 1. Effect of different concentrations of γ-butyrolactone on the P(3HB-co-4HB) production of *Cupriavidus eutrophus* B10646 under heterotrophic (substrate – butyric acid) conditions

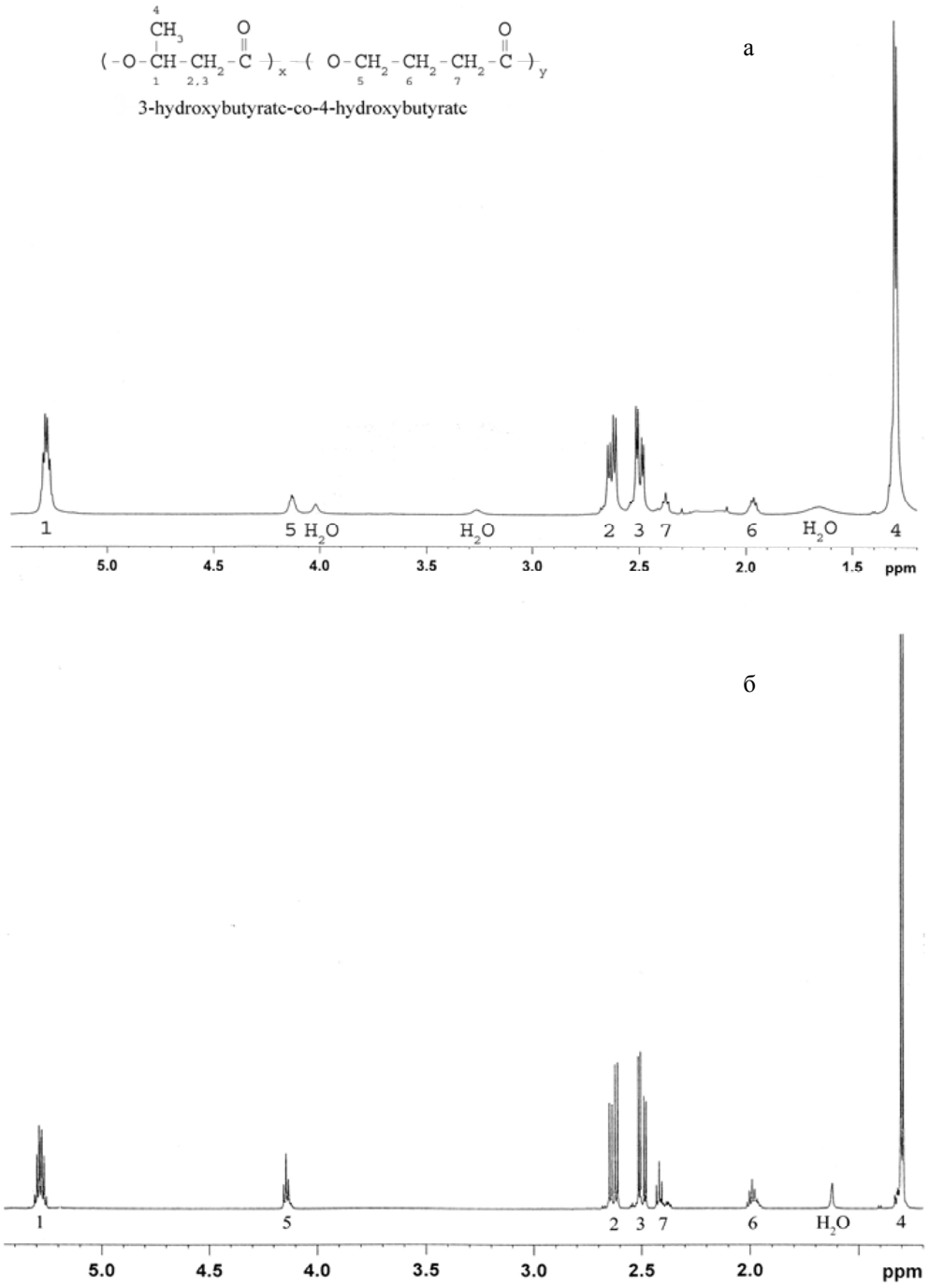

Fig. 2. ^{1}H-NMR spectrum of the P(3HB-co-4HB) with content of 4HB 8.7 mol % (a) and 16 mol % (b)

of γ-butyrolactone in the culture provided significantly higher values of the three key indexes: biomass yield, copolymer concentration in cells and content of 4HB.

The properties of the set of synthesized and highly purified samples of P(3HB-*co*-4HB) were studied using modern physical methods. Using the gel permeation chromatography, the molecular mass of the set of the synthesized samples of copolymers P(3HB-*co*-4HB) was determined (Table 2). No influence of this parameter on the molecular mass and the polydispersity of the copolymer was detected in the studied range of the monomers ratio in the copolymer (the incorporation of 4HB varied from 8.7 to 24.3 mol %). The number average mass in the set of samples varied in the range from 230 to 480 kDa, the weight average molecular mass amounted to 540–1110 kDa; the polydispersity varied from 1.91 to 2.76 with no clear correlation with the content of 4HB in the copolymer.

It has been demonstrated that the incorporation of 4HB influenced significantly the ratio of crystalline and amorphous phases in the copolymer (to the higher extent if compared to 3-hydroxyvalerate and 3-hydroxyhexanoate), sufficiently reducing its crystallinity (Table 2). For the first time we managed to obtain the samples of PHA with the lowered degree of crystallinity (from 44 % to 12 %).

The melting temperature of the produced samples of P(3HB-*co*-4HB) was lower (168.9-172.5ºC) in comparison to poly(3-hydroxybutyrate) (180ºC). However, no relation between the 4HB content in the range from 8.7 mol % to 17 mol % and the melting temperature has been determined. The temperature of thermal degradation of various copolymer samples varied in the range from 264ºC to 286ºC and did not depend on the content of 4HB.

Discussion

Analysis of the up-to-date literature witnesses the activity of the research aimed at the synthesis of copolymer P(3HB-*co*-4HB). This type of PHA characterized by high elasticity and strength, as well as quicker and regulated speed of biodegradation, attracts the attention of the researchers who develop the products intended for the biomedical field. For the present moment the company Metabolix (USA) has developed and produced a line of products made out of this copolymer in the form of suture material, films and reticular implants. For successful production of this type of PHA many research teams isolate new strains-producers, improve the fermentation technology using various compounds as growth

Table 2 Physical characterization of P(3HB-co-4HB) produced by *Cupriavidus eutrophus* B10646

4HB content, mol %	M_n (× 10^3) Da	PD	Crystallinity, %	Melting temperature (T_m) , ºC	Degradation temperature (T_d), ºC
0	760 ± 15	1.60 ± 0.03	76	180	280
8.7	230 ± 2	2.76 ± 0.03	44	171	286
10.7	480 ± 9	2.32 ± 0.06	43	171.9	268
14.9	320 ± 7	2.65 ± 0.04	44	168.9	264
16.0	370 ± 5	2.59 ± 0.04	43	171.3	279
17.0	370 ± 12	2.31 ± 0.06	25	172.5	271
24.3	285 ± 5	1.91 ± 0.01	12	-	274

- not identified

substrates. Thus, Li with co-authors (2010) has determined that the recombinant strain *E. coli* containing the plasmid with the genes incorporated into the succinate degradation out of *Clostridium kluyveri*, and the directional genes of the poly(3-hydroxybutyrate) synthesis out of *R. eutropha,* can synthesize with higher yields (up to 70 % of DCW) of copolymer P(3HB-*co*-4HB). Among the natural producers various bacteria have been described. Recently new highly productive strains have been isolated from soil in Thailand and bottom sediments of the lake in Malaysia which have been identified correspondingly as *R. eutropha* A-04 (Chanprateep et al., 2008) and *Cupriavidus* sp. USMAA1020 (Amirul et al., 2008), synthesizing copolymers P(3HB-*co*-4HB) using γ-butyrolactone, 1,4-butanediol or 4-hydroxybutyrate acid. If *R. eutropha* A-04 is grown on 4-hydroxybutyric acid the maximum incorporation of 4HB amounts to 52 mol % with 22 % of polymer of DCW biomass. The maximum content of the copolymer (59 % of the dry biomass) was obtained using the mixture of 4-hydroxybutyric acid and butyric acid (1:1), nevertheless, the incorporation of 4HB did not exceed 24 mol % (Chanprateep et al., 2008). When the strain *Cupriavidus* sp. USMAA1020 was grown on γ-butyrolactone, 4- hydroxybutyric acid and 1,4-butanediol, the best substrate for growth and synthesis of the polymer (4.96 g/L and 58.7 % of DCW, correspondingly) for this strain was 1,4-butanediol with 32 mol % of 4HB (Amirul et al., 2008). Higher values of 4HB in the copolymer (over 80 mol %) were obtained using the mixture of 1,4-butanediol and γ-butyrolactone, nevertheless, the biomass and the polymer content reduced therefore to 1.9 g/L and 16 % of the dry weight (Vigneswari et al., 2010). The copolymer with the same high content of 4HB was produced during the cultivation of another strain of bacteria *Wautersia eutropha* H16 in the medium which besides 4-hydroxybutyric acid also contained amino acids L-alanine and L-threonine, however, the biomass yield was significantly higher, about 12 g/L, but the content of the polymer did not exceed 42 % of DCW (Kimura et al., 2008). Moreover, Nakamura and co-authors (1992) proved the possibility to produce homopolymer P(4HB), but the content of homopolymer did not exceed 2 %. To reduce the cost of the polymer the research on using cheaper carbon sources for the copolymer P(3HB-*co*-4HB) synthesis have been carried out, including oleic acid and saturated fatty acids with even amount of carbon atoms (C12-C18) (Rahayu et al., 2008; Ramachandran et al., 2011), alkanediols (Vigneswari et al., 2009), worked out palm oil (Rao et al., 2010), soy bean oil (Park, Kim, 2011), nevertheless, no significant yields of the copolymer P(3HB-*co*-4HB) on these substrates have been obtained.

Thus, using the recently produced, new natural strains and recombinant producers the possibility of synthesizing the copolymer P(3HB-*co*-4HB) with different yields (from 2 % to 70 % of DCW) of the copolymer with the content of 4HB up to 100 mol % at various yields of the biomass yield (from 2 to 12 g/L) has been determined.

In this work the results of studying the synthesis of the copolymer P(3HB-*co*-4HB) by two natural strains of hydrogen-oxidizing bacteria are presented. The analysis of the capability of the strain *R. eutropha* B5786 characterized by the high yields of poly(3-hydroxybutyrate) (up to 90 % of DCW) at different substrates including the synthesis-gas (Volova, Voinov, 2004) and the copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (Volova et al., 2007) and 3- and 4-component PHA formed by the monomers C4, C5, C6, C7 (Volova et al., 2006) has shown that content of the monomer in the copolymer did not exceed 4 mol %, the maximum biomass production and copolymer content amounted for 6 g/l and 60 % of DCW, correspondingly, due to the pronounced inhibiting effect of γ-butyrolactone

on this strain. Attempts to change the dosage mode of γ-butyrolactone (fractional adding) allowed to increase the total biomass and the copolymer production, unfortunately, the content of 4HB did not exceed 6 mol % as in autotrophic, so in heterotrophic conditions.

As shown in the presented work, for the strain *R. eutropha* B5786 with concentration of γ-butyrolactone exceeding 6 g/L in the culture, the decrease of the yield and copolymer production is observed due to the inhibiting effect. Another studied strain *C. eutrophus* B10646 selected for the sustainability to γ-butyrolactone influence (registered in the collection of the Russian Collection of Industrial Microorganisms in 2010) allowed us to obtain higher results. Unlike the strain *R. eutropha* B5786, γ-butyrolactone with concentration up to 15 g/L in the medium did not inhibited the growth and the synthesis of the polymer in the strain *C. eutrophus* B10646. The fulfilled mode of growing allowed to obtain good results in the biomass production (to 7-8 g/L of 72 hours of cultivation) with the yield of copolymer up to 80-90 % of DCW and the content of 4HB monomer up to 17 mol %. These results can be compared with the data of the other research obtained by Amirul et al. (2008) during the cultivation of the strain *Cupriavidus* sp. USMAA1020 and Chanprateep et al. (2008) during the cultivation of the strain *R. eutropha* A-04.

The samples synthesized in the experimental amounts with the different ratio of monomers 3HB and 4HB purified to the homogeneous condition allowed to study their properties: molecular mass, degree of crystallinity and temperature characteristics. At the same time no relation between the 4HB content and the copolymer's molecular mass has been determined. This confirms the previous results that there is no correlation between the composition of PHA and the molecular mass (Volova, Kalacheva, 2005). The absence of correlation between the molecular weight of the polymer and the 4HB content has been also demonstrated for *Comamonas acidovorans* (Mitomo et al., 2001). Nevertheless, Vigneswari et al. (2009) has demonstrated the inverse dependence between the 4HB content and the molecular mass for *Cupriavidus* sp. USMAA1020**.**

The crystallinity degree of the copolymers P(3HB-*co*-4HB) with the content of the 4HB ranging from 8.7 to 16 mol % amounted to 43-44 % which corresponds to the results of other authors (Doi et al., 1990; Nakamura et al., 1992; Tsuge, 2002). Thus, Mitomo et al. (2001) has demonstrated that the crystallinity degree of the copolymer with the 4HB content of 19, 38 and 65 mol % amounted for 40, 18 and 27 %, correspondingly. The authors have assumed that the crystallinity degree of the copolymer is minimum when the 4HB content is 40-50 mol % (Mitomo et al., 2001). Nevertheless, according to the data of the other authors the minimum crystallinity degree of 17-18 % has been determined for the polymers with the 4HB content of 78-82 mol % (Saito, Doi, 1994).

There is uncertainty of the obtained data in the literature for the melting temperature of the P(3HB-*co*-4HB) samples. According to the data of some authors the melting temperature of the samples with 4HB content of 7 mol %, 11 mol % and 15 mol % amounted to 173, 169 and 160.8ºC correspondingly (Kunioka et al., 1989; Doi, 1990; Rao et al., 2010), when in the other papers the melting temperature of the samples with the similar amount of 4HB is considerably lower: 114ºC and 131.5ºC (Xie, Chen, 2008; Li et al., 2010). In our work the melting temperature for the set of P(3HB-*co*-4HB) samples amounted to 168-171.9 °C. The gap between the melting temperature and the temperature of thermal degradation for the set of the studied samples made 95.1-115ºC. We

have not found any references regarding the temperature of thermal degradation of P(3HB-*co*-4HB).

Thus, P(3HB-co-4HB) was produced by *R. eutropha* B5786 and *C. eutrophus* B10646 under autotrophic and heterotrophic conditions using γ-butyrolactone as source of 4HB. It was shown that *C. eutrophus* B10646 possesses the increased sustainability to the γ-butyrolactone influence. The fulfilled mode of growing of *C. eutrophus* B10646 allowed to obtain good results in the biomass production (to 7-8 g/L of 72 hours of cultivation) with the yield of copolymer up to 80-90 % of DCW and the content of 4HB monomer in it to 17 mol %). A set of highly purified samples of P(3HB-*co*-4HB) with different content of 4HB was produced and their physicochemical properties were studied.

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Микробный синтез и характеристика сополимеров поли(3-гидроксибутирата-со-4-гидроксибутирата)

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Работа посвящена перспективному представителю семейства биоразрушаемых полигидроксиалканоатов (ПГА) сополимеру поли(3-гидрокмсибутирата-со-4-гидроксибутирата) (поли(3ГБ-со-4ГБ)). Трудности получения этого типа ПГА связаны с тем, что предшественник для синтеза мономеров 4-гидроксибутирата (4ГБ) – γ-бутиролактон токсичен для бактерий-продуцентов и при его добавлении в среду снижаются общие выходы биомассы и полимера. С использованием природных штаммов водородокисляющих бактерий Ralstonia eutropha B5786 и Cupriavidus eutrophus B10646 последний обладает повышенной

устойчивостью к воздействию γ-бутиролактона, найдены условия культивирования для эффективного синтеза поли(3ГБ/4ГБ). Получена серия высокоочищенных образцов поли(3ГБ/4ГБ) с различным содержанием 4ГБ (от 8,7 до 24,3 мол. %). Установлено, что включение 4ГБ в сополимер в большей степени, нежели 3-гидроксивалерат и 3-гидроксигексаноат, приводит к снижению кристалличности сополимера; получены образцы, имеющие степень кристалличности 12 и 25 %. Показано, что средняя молекулярная масса образцов поли(3ГБ/4ГБ) и полидисперсность не зависят от соотношения мономеров и варьируют в широких пределах, соответственно, от 540 до 1110 кДа и от 1,91 до 2,76. Установлено, что содержание 4ГБ в исследуемом диапазоне (8,7-24,3 мол. %) не влияло на температуру плавления сополимеров (168,9-172,5 ºС).

Ключевые слова: водородокисляющие бактерии; поли(3-гидроксибутират-со-4-гидроксибутират); γ-бутиролактон; физико-химические свойства.